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Pollen fertility restoration by nuclear gene *Fr* in CMS common bean: an *Fr* linkage map and the mode of *Fr* action

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Abstract The *Fr* gene in common bean, *Phaseolus vulgaris L.*, is a unique gene for the study of plant nuclear-mitochondrial interactions because it appears to directly influence plant mitochondrial genome structure, resulting in the restoration of pollen fertility in cytoplasmic male sterile plants. This gene action is distinct from other pollen fertility restoration systems characterized to date. As a first step towards the map-based cloning of this unusual nuclear gene, we identified RAPD markers linked to *Fr* using bulked segregant analysis of near-isogenic lines. Using DNA gel blot hybridization, we localized the identified RAPD markers to a linkage group on the common bean RFLP map and constructed a linkage map of the *Fr* region using both RAPD markers and RFLP markers. Analysis of the mode of *Fr* action with the aid of identified *Fr*-linked DNA markers indicated that *Fr* functions in a semidominant fashion, showing dosage effect in controlling the dynamics of a heteroplasmic mitochondrial population. We also present our observations on the developmental distinctions, crucial in the accurate mapping of the *Fr* gene, between spontaneous cytoplasmic reversion and *Fr*-driven fertility restoration, two phenomena that are phenotypically indistinguishable.

Key words *Phaseolus vulgaris* L. · *Fr*-directed mitochondrial alteration · *Fr* linkage map · Semidominant effect · Map-based cloning

Introduction

Cytoplasmic male sterility (CMS) and its nuclear fertility restoration genes are among the few nuclear-mitochondrial genetic interaction systems in plants amenable to in-depth investigation (Hanson 1991). In many plant species the CMS phenotype, which involves a maternally-inherited failure to shed viable pollen, is caused by a mitochondrial lesion. In most CMS systems investigated to date, nuclear restorer genes have been identified that suppress the expression of the mitochondrial sterility-associated lesions, resulting in restoration to pollen fertility (Hanson and Conde 1985). These CMS-restorer systems are ideal for the investigation of plant nuclear-mitochondrial interactions. Unfortunately, little is yet known about the mechanisms of fertility restoration in even the best characterized CMS systems.

Fertility restoration by nuclear gene *Fr* in CMS common bean is unique when compared to other known CMS systems. The majority of fertility restorer genes appear to regulate gene expression at a transcriptional or posttranscriptional level (Kennell et al. 1987; Pruitt and Hanson 1991; Singh and Brown 1991; Laver et al. 1991; Iwabuchi et al. 1993). *Fr* restores fertility by causing the elimination of the sterility-associated *pvs* (*Phaseolus vulgaris* sterility) DNA sequence from the mitochondrial genome, resulting in irreversible and permanent restoration (Mackenzie et al. 1988; Mackenzie and Chase 1990). Therefore, elucidation of the mechanism of *Fr* action can lead us to understand how the plant nuclear genome maintains a normal mitochondrial genome structure by the selective elimination of mitochondrial mutations.

Spontaneous cytoplasmic reversion to fertility presents another feature unique to CMS common bean (Mackenzie et al. 1988). Unlike the well-documented spontaneous cytoplasmic reversion in CMS-S maize (Laughnan et al. 1981; Small et al. 1988), CMS bean reversion events involve the complete loss of a portion of the mitochondrial genome. This mitochondrial DNA (mtDNA) loss is indistinguishable from the mitochondrial genome changes induced by nuclear gene *Fr* and appears to involve the loss of an entire mitochondrial chromosome (Janska and Mackenzie 1993).

Our objective is to clone the *Fr* locus. Cloning such a gene, however, represents a substantial technical challenge

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because neither is the gene product known nor have similar genes been isolated from other organisms. Consequently, a map-based cloning strategy presents the most feasible approach for isolating *Fr*. This is because common bean has a relatively small genome (637 Mbp or 0.66 pg/1C) (Arumuganathan and Earle 1991), and two genetic linkage maps of the common bean genome are available with over 400 restriction fragment length polymorphic DNA (RFLP) markers (Vallejos et al. 1992; Nodari et al. 1993). The first step in a map-based cloning approach is the development of a dense linkage map around the target gene. Accordingly, we have used DNA markers to determine the genomic location of *Fr* in reference to mapped DNA markers in a two-step process. First, we used a strategy that combines bulked segregant analysis (Michelmore et al. 1991) and near-isogenic lines (NIL) (Martin et al. 1991) to identify random amplified polymorphic DNA (RAPD) markers tightly linked to the *Fr* locus. We then used segregation and linkage analyses to map *Fr* and identify additional linked marker loci. The establishment of the *Fr* linkage map with closely linked markers not only facilitates progress toward our long-term objective of cloning the gene but also enables us to further characterize the mode of gene action of *Fr*.

Materials and methods

Plant materials

Three segregating populations were used in this study. (1) A BC_6F_2 population (designated R351 BC_6F_2), which was derived from a cross between *Phaseolus vulgaris* lines CMS-Sprite (*frfr*) and R351 (*FrFr*) using CMS-Sprite as the recurrent parent. CMS-Sprite contains the sterility-inducing cytoplasm (characterized by the presence of *pvs* in the mitochondrial genome) and a 'Sprite' nuclear background. The development of the CMS line and the restorer line R351 has been described previously (Mackenzie and Bassett 1987). The R351 BC_6F_2 population consisted of 47 plants. This population was used for bulked segregant analysis to identify *Fr*-associated RAPD markers. (2) A BC_4F_2 population (designated R351 BC_4F_2) was derived from a different cross but using the same parents of R351 BC_6F_2. This population contained 81 plants and was used to map the *Fr* locus relative to identified RFLP markers and RAPD markers. (3) A BC_1 population of 68 plants was derived from a cross between the Meso-American breeding line XR235-1-1 and the Andean cultivar 'Calima' as described by Vallejos et al. (1992). This was the same population that was used to construct the common bean genetic linkage map and was used to localize the *Fr*-linked RAPD markers in this study.

Phenotypic classification of male fertility

Plants were grown under standard greenhouse conditions and classified for pollen fertility using criteria described by Mackenzie and Bassett (1987) with modifications. Fertile plants produced greater than 90% fertile pollen staining darkly with I-KI and produced normal seed-bearing pods with no evidence of parthenocarpy. Sterile plants shed no visible pollen on the stigma, and the nonstainable aberrant microspores were bound together as tetrads, producing only parthenocarpic pods bearing no seeds. Semisterile plants produced both dark-staining fertile pollen and nonstainable aberrant tetrads in each anther, giving rise to fertile and sterile buds and resulting in both seed-bearing and parthenocarpic pods on an individual plant.

DNA extraction

Genomic DNA was extracted from young leaf tissue using a modified procedure of Vallejos et al. (1992). About 2 g of tissue were ground in liquid N_2 to a very fine powder and incubated with 14 ml lysis buffer (133 m*M* TRIS-HCl (pH 7.8), 6.7 m*M* Na_2EDTA, 0.95 *M* NaCl, 1.33% Na Sarkosyl and 1.33% β-mercaptoethanol) at 65 °C for 1 h. The homogenate was chloroform-extracted once and the aqueous phase separated by centrifugation. DNA was then precipitated in 2/3 volume of isopropanol at room temperature for about 1 h. Precipitated DNA was then transferred with a glass hook to a new tube, dissolved in 3 ml TE (10 m*M* TRIS, pH 8, 1 m*M* Na_2EDTA) and incubated with 60 µg RNAase at 37 °C for 30 min. The solution was chloroform-extracted once more, and DNA was reprecipitated by the addition of 1/20 volume of 5 *M* ammonium acetate and 2 volumes of absolute ethanol. Precipitated DNA was scooped, air-dried briefly and dissolved in 500 µl TE buffer.

RAPD marker screening

Genomic DNA from the R351 BC_6F_2 plants was used as template for the polymerase chain reaction (PCR). Two pairs of DNA bulks were formed for bulked segregant analysis. One pair contained 10 fully fertile plants and 10 fully sterile plants in the contrasting bulks, and the other pair contained only 2 fertile plants and 2 sterile plants in the contrasting bulks. Decamer oligonucleotide primers were obtained from the University of British Columbia, Vancouver, Canada. Custom-made primers were synthesized in the Biochemistry Department at Purdue University. A single primer was used in each PCR reaction. About 700 primers were assayed. Amplification reactions were in total volumes of 30 µl containing 50 m*M* KCl, 10 m*M* TRIS-HCl, 2.0 m*M* $MgCl_2$, 0.1% Triton X-100, 125 µ*M* each of dATP, dCTP, dGTP and dTTP, 1 µ*M* primer, 100–200 ng genomic DNA and about 0.7 unit *Taq* DNA polymerase (Promega). Amplification was performed in a Thermolyne Temp-Tronic Thermal Cycler (Barnstead/Thermolyne Co.) for 40 cycles after initial denaturation at 92 °C for 3 min. Each cycle consisted of 45 s at 94 °C, 1 min 15 s at either 37 °C or 43 °C, 1 min 20 s at 72 °C, followed by 7 min at 72 °C. Amplifications were resolved by gel electrophoresis in a 1.2% agarose gel and visualized by staining with ethidium bromide. Putative polymorphic amplifications were verified using genomic DNAs from 5 of each fertile and sterile individual plants as templates to assure cosegregation of the polymorphism with fertility. Confirmed *Fr*-linked markers were then applied to the entire population for genetic mapping.

RFLP analysis

Genomic DNA from R351 BC_4F_2 plants and the XR235-1-1/'Calima' BCF_1 population was used for RFLP analysis. DNA samples were digested with restriction endonucleases (*Hin*dIII, *Pst*I, *Bam*HI, *Eco*RI, *Eco*RV and *Dra*I) using reaction conditions recommended by the manufacturers, followed by electrophoretic separation in 0.8% agarose gel using 1×TBE buffer. DNA was affixed to the nylon membrane (Hybond-N, Amersham) by UV cross-linking and/or baking for 2 h (80 °C) under vacuum. RFLP clones were amplified using the procedure described by Vallejos et al. (1992) and purified using the Magic purification kit (Promega). DNA fragments were radioactively labeled with [^{32}P]dCTP using the random priming method (Feinberg and Vogelstein 1983). Hybridization was performed at 60°C. Blots were washed twice in 3×SSC, 0.1% SDS at 60 °C and once in 0.3×SSC at 60 °C for 15 min each wash. Fuji Medical X-Ray film was exposed to the membrane using intensifying screens (Pickett) for 5–7 days for nuclear DNA probes and 1–2 days for mtDNA probes.

Segregation and linkage analysis

Allelic segregation data were scored manually from either ethidium bromide-stained agarose gels (RAPDs) or autoradiographic films

(RFLPs) for each segregating individual. Linkage analysis was performed using the Mapmaker program (Lander et al. 1987). The Kosambi mapping function (Kosambi 1944) was used in calculating genetic distances. The map order was based on maximum likelihood estimation. Two-point analysis was used to determine genetic distances between markers.

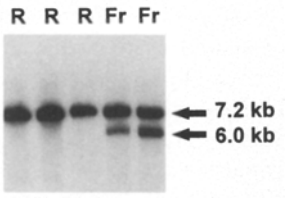

Fig. 1 DNA gel blot hybridization of total genomic DNA showing that the sterility-associated mitochondrial *pvs* DNA sequence is present in the vegetative tissue of *Fr*-restored F_2 fertile plants but is lost in the vegetative tissue of spontaneous cytoplasmic fertile revertants. The total genomic DNA was prepared from green leaf tissue, digested with *Pst*I, and the blot was hybridized with a mtDNA clone (258-1) that is internal to the 6.0-kb *pvs Pst*I fragment and a 7.2-kb fragment residing elsewhere in the mitochondrial genome (Mackenzie et al. 1988). *Fr* R351 BC_4F_2 fertile *Fr*-restored plant, *R* R351 BC_4F_2 spontaneous fertile revertant

Results

Assessment of the populations segregating for *Fr*

The major objective of this project was to determine the linkage relationships of *Fr* and establish a dense map of the *Fr* region. Thus, it was essential to identify with accuracy the phenotype conferred by *Fr*, and an intrinsic problem of phenotypic misclassification with our mapping populations must be circumvented. In CMS-common bean it has been demonstrated that pollen fertility restoration through the deletion of the sterility-inducing mitochondrial *pvs* sequence can be achieved by either the introduction of nuclear gene *Fr* or a spontaneous cytoplasmic reversion event. Both events result in the same fertile phenotype and apparently identical mitochondrial genome alterations (Janska and Mackenzie 1993). The frequency of spontaneous cytoplasmic reversion depends on both genetic background and growing conditions (especially temperature). Spontaneous cytoplasmic reversion of CMS-Sprite occurs at a frequency of 0.5%–5% (He and Mackenzie unpublished).

The R351 BC_6F_2 population that consisted of 47 plants was used for bulked segregant analysis to identify RAPD markers linked to *Fr*. Spontaneous cytoplasmic reversion was not considered a problem in this population because of its low frequency of occurrence. The population segregated in a Mendelian fashion ($\chi^2_{1:2:1}$=0.732, P=0.69).

The R351 BC_4F_2 population was used for linkage analysis. Therefore, precautions were taken to ensure an accurate assessment of the genotype at the *Fr* locus to avoid misinterpretation of mapping data due to spontaneous reversion events. Fortunately, we were able to distinguish reversion from *Fr*-driven restoration based on the fact that the *pvs* sequence persists in the vegetative tissue of fertile F_2 plants that have had their fertility restored by *Fr* (Johns et al. 1992). A survey of fertile plants in the R351 BC_4F_2 mapping population revealed 6 plants missing the *pvs* sequence in their vegetative tissue (Fig. 1); these results suggested that the six plants were spontaneous revertants and could possess the *frfr* genotype. The genotype of these plants was confirmed by genetic analysis. Three of the putative revertants (*frfr*), along with an *Fr*-restored fertile plant (*FrFr*) and a semisterile plant (*Frfr*), were crossed to CMS-Sprite. Spontaneous reversion to fertility is a cytoplasmic event that does not require the introduction of any nuclear fertility restorer gene; therefore, when it is crossed as pollinator to CMS-Sprite all resulting F_1 progeny should be sterile. When an *Fr*-restored fully fertile plant is crossed to CMS-Sprite, all resulting progeny are expected to be semisterile. Accordingly, when a semisterile plant is crossed to CMS-Sprite, half of the resulting F_1 progeny are expected to be semisterile and half sterile. Table 1 shows an example of testcross results which confirms that plant 16-19-3, a fertile F_2 plant that had lost the *pvs* sequence from its vegetative tissue, contained no nuclear restorer gene, and therefore is a spontaneous revertant with genotype *frfr*. The distinction of spontaneous cytoplasmic revertants led to a reclassification of the population ($\chi^2_{1:2:1}$=2.92; P=0.25).

Table 1 Verification of genotypes at the *Fr* locus predicted by *Fr*-linked markers

Cross[a]	Phenotype of pollen donor	Genotype of *Fr*-linked marker[b]	Segregation in F_1 plants		Genotype at *Fr* locus
			Semisterile	Sterile	
CMS-Sprite/ 16-19-3	Fertile	*aa*	0	17	*frfr*
CMS-Sprite/ 2-8-5	Fertile	*AA*	13	2[c]	*FrFr*
CMS-Sprite/ 16-17-7	Semisterile	*Aa*	12	12	*Frfr*

[a] CMS-Sprite is an accession line containing sterility-inducing cytoplasm and the *frfr* nuclear genotype. The pollen donors 16-19-3, 2-8-5 and 16-17-7 were R351 BC_4F_2 segregants

[b] *A* represents the marker allele coupled to the *Fr*-restored fertile phenotype; *a* represents the marker allele coupled to *fr*

[c] The 2 plants failed to set any seed pod. This was likely due to environmental stress

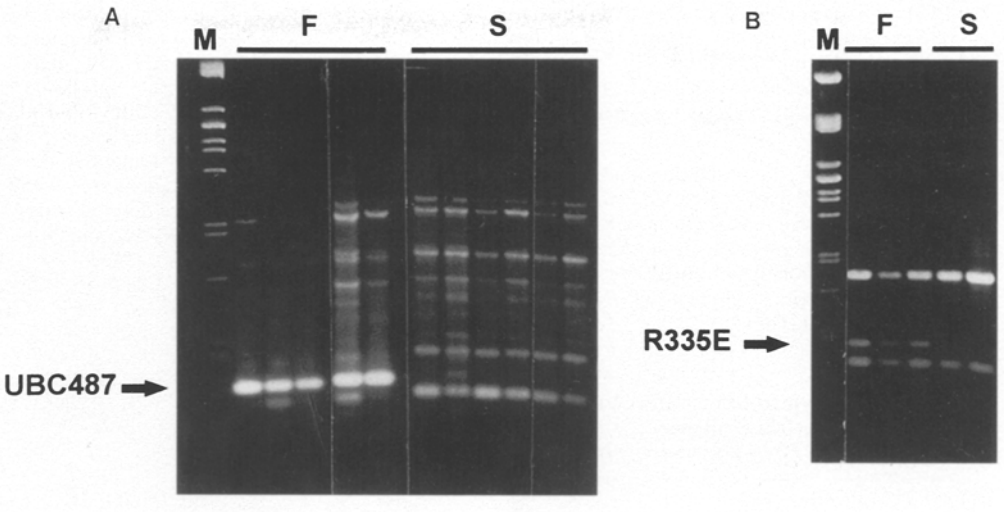

Fig. 2A, B Identification of RAPD markers associated with the *Fr* locus. **A** Cosegregation of RAPD marker *UBC487* with pollen fertility specified by the *Fr* gene. Each lane represents the amplification of an individual fertile (F) or sterile (S) R351 BC_6F_2 plant using primer *UBC487*. The *arrow* indicates the polymorphic band associated with fertile plants. *M lambda PstI* molecular weight marker. **B** Conversion of the highly repetitive DNA sequence of RAPD marker *UBC487* to a low-copy-number marker *R335E*. The *arrow* points to the new polymorphic band that is present in fertile (F) plants but absent in sterile (S) plants

Table 2 Decamer primer sequences that produce *Fr*-linked polymorphic amplifications

Primer	Sequence	Polymorphism
UBC487	5′ GTGGCTAGGT 3′	Dominance
R335E	5′ ACCTAGCCAC 3′	Dominance
UBC326	5′ CGGATCTCTA 3′	Co-dominance
UBC375	5′ CCGGACACGA 3′	Dominance

Identification of RAPD markers linked to the *Fr* locus

A strategy that combines bulked segregant analysis (Michelmore et al. 1991) and near-isogenic line analysis (Martin et al. 1991) was employed to identify RAPD markers linked to the *Fr* locus. Two experiments were undertaken with different DNA bulks and primer annealing temperatures. In the first experiment, the contrasting bulked DNA samples were formed separately from the DNA of 10 R351 BC_6F_2 *Fr*-restored fully fertile plants and 10 sterile plants. These were used as DNA templates to screen decamer primers for DNA polymorphisms under the conditions described in the Materials and methods, using 43C as primer annealing temperature. In the second experiment, only DNA from 2 fertile and 2 sterile plants was included in the contrasting bulks, and 37C was used for primer annealing. Altogether, about 5,000 loci were amplified with 700 primers, and 3 demonstrated polymorphic amplifications that cosegregated with pollen fertility when they were tested in a number of individual fertile and sterile R351 BC_6F_2 plants. The primer identities and types of amplification are listed in Table 2. Primers *UBC487* and *R335E* were used in amplification at the 43C primer annealing temperature, whereas primers *UBC326* and *UBC375* were used at an annealing temperature of 37C. The primer codes are henceforth used to designate the corresponding amplified RAPD markers. *UBC487*, *UBC326* and *R335E* demonstrated complete dominance and were linked in coupling with the *Fr* allele, whereas *UBC375* showed codominance with *Fr* and *fr* alleles. Figure 2A shows an example of the cosegregation of a RAPD marker (*UBC487*) with fertility restoration.

Genomic localization of the *Fr*-linked RAPD markers

The second step of our mapping effort consisted of determining the position of the *Fr*-linked RAPD markers in the RFLP map of common bean. The objective of this effort was to identify additional markers around the *Fr* locus. Towards this end, amplification products linked to *Fr* were removed from the gel and used as hybridization probes to identify restriction enzymes that revealed polymorphisms between the parents of the RFLP mapping population ('Calima' and XR235-1-1). Unfortunately, all three fragments yielded hybridization patterns typical of repetitive DNA (data not shown). To circumvent this difficulty, we developed a "reverse primer" approach; primers complementary but in opposite orientation to *UBC487*, *UBC326* and *UBC375* were synthesized and used as single primers for RAPD analysis. Two of these failed to produce polymorphic fragments. However, one reverse decamer primer, designated *R335E* and complementary to *UBC487*, produced a polymorphic fragment cosegregating with the *Fr* locus (Fig. 2B). As with *UBC487*, no recombinants between this fragment and *Fr* were detected in either the R351 BC_4F_2 or R351 BC_6F_2 populations with a total number of 125 plants assayed. Furthermore, when the polymorphic fragment amplified with *R335E* was used as a hybridization probe on DNA gel blots, it produced a hybridization pattern of a sequence with a lower reiteration frequency than the one observed with the *UBC487* fragment. Thus, *R335E* appears to direct the amplification of a less repetitive sequence than the adjacent sequence amplified with *UBC487*.

The polymorphic *R335E* fragment was used as a probe to identify a restriction enzyme that revealed polymor-

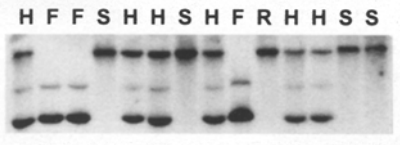

Fig. 3 DNA gel blot hybridization analysis demonstrating the cosegregation of RFLP marker *Bng228* with pollen fertility effected by the *Fr* gene. Each lane represents an individual plant from the R351 BC_4F_2 population segregating at the *Fr* locus. The *Eco*RI-digested DNA gel blot was probed with *Bng228*. *F* Fertile plant (*FrFr*), *S* sterile plant (*frfr*), *H* semisterile plant (*Frfr*), *R* spontaneous cytoplasmic fertile revertant (*frfr*)

Table 3 DNA polymorphisms detectable between two NILs of the *Fr* locus using nine RFLP clones residing in the chromosomal region encompassing *Fr* (+ polymorphic band detected)

RFLP clone	Enzyme					
	*Hin*dIII	*Pst*I	*Bam*HI	*Eco*RI	*Eco*RV	*Dra*I
Bng5	+				+	
Bng14	+			+		
Bng19						
Bng20						
Bng24	+		+	+		
Bng51						
Bng64				+	+	
Bng102				+		
Bng228		+		+		+

phisms between 'Calima' and XR235-1-1, the progenitors of the population used to construct the RFLP linkage map of common bean. Next, DNA gel blots of *Eco*RI digests of the BC_1F_1 (XR235-1-1//XR235-1-1/'Calima') were hybridized to *R335E*, and the segregation of a single fragment was recorded. The segregation of this fragment was tested against those of all other previously mapped RFLP markers (Vallejos et al. 1992) with the aid of Mapmaker (Lander et al. 1987). The segregating fragment was located in the vicinity of *Bng228-Bng102* on linkage group K (data not shown). We then used a separate mapping population (R351 BC_4F_2), segregating at the *Fr* locus, to verify this map location and establish the map site of the *Fr* locus. RFLP marker *Bng228* of linkage group K was used to probe a genomic DNA gel blot of 14 individual plants from this population. The marker cosegregated with pollen fertility as is clearly evident in Fig. 3. This R351 BC_4F_2 population of 81 plants was subsequently used for constructing an *Fr* linkage map.

Construction of a linkage map of the *Fr* region

Nine RFLP markers from linkage group K were used to screen for polymorphisms between a male-fertile and a male-sterile plant in the R351 BC_4F_2 population. As shown in Table 3, six of the nine clones demonstrated numerous polymorphisms between the two NILs using six different restriction endonucleases (*Hin*dIII, *Pst*I, *Bam*HI, *Eco*RI, *Eco*RV and *Dra*I). It is expected that, after four generations of backcrossing and one terminal selfing generation to develop the R351 BC_4F_2 population, greater than 97% of the recurrent parent genome was recovered, with the remnant donor parent DNA limited primarily to the chromosomal segment surrounding the introgressed marker *Fr* (Muehlbauer et al. 1988). Consequently, the numerous polymorphisms in this population detected with RFLP markers in the linkage group K support the assumption that *Fr* is located in the K linkage group.

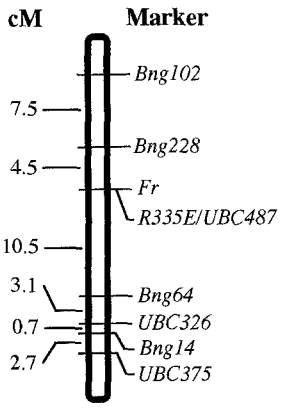

Fig. 4 *Fr* genetic map showing the location of the *Fr* locus and linked RFLP and RAPD markers. The map order was based on maximum likelihood estimations using the Mapmaker computer program (Lander et al. 1987). All distances are given as cM and were derived from the R351 BC_4F_2 mapping population

Informative probe-restriction enzyme combinations were used to study the segregation of the *R335E*-linked RFLP markers in the R351 BC_4F_2 progeny. *Eco*RI-digested genomic DNA gel blots were used to score RFLP markers *Bng14*, *Bng64*, *Bng102* and *Bng228* from linkage group K. *Pst*I-digested DNA gel blots were also used in mapping *Bng228*. This same population was employed to assay the segregation of RAPD markers *UBC487(R335E)*, *UBC326* and *UBC375*. The fertility phenotype was classified so that fertile and semisterile plants were grouped together (*F_*) to avoid misclassifications between the two classes. The resulting *Fr* linkage map based on maximum likelihood estimation using three RAPD markers and four RFLP markers is shown in Fig. 4.

The overall genetic distances among the markers on this linkage map are greater than those reported in the original map developed by Vallejos et al. (1992). An extreme example is the distance between *Bng14* and *Bng228*; no recombination was evident between these markers in the original mapping population, but they are separated in our map by up to 19 cM, suggesting that recombination suppression occurred in this chromosomal region within the original mapping population.

Some of the markers identified are much more closely linked to *Fr* than others. It should be mentioned that two RAPD markers, *UBC375* and *UBC326*, were identified by screening RAPD primers with two contrasting DNA bulks that contained only 2 fertile and 2 sterile R351 BC_6F_2 near-isogenic plants. These markers mapped 15 cM and 21 cM, respectively, from the *Fr* locus. On the other hand, marker *UBC487* was identified by bulking 10 fertile and 10 sterile plants as the contrasting bulks and it demonstrated complete cosegregation with the *Fr* allele in 125 plants assayed

Table 4 Contingency table for the distribution of the *Bng228* marker genotype and fertility in a R351 BC4F2 populationa

Genotype at *Bng228* locus	Fertility			
	Fertile	Semisterile	Sterile	Total
AA	11	1	0	12
Aa	2	39	0	41
aa	0	4	18	22
Total	13	44	18	75

χ^2 (1*AA*: 2*Aa*: 1*aa*) = 3.32 $P = 0.18$
χ^2 (1 fertile:2 semisterile:1 sterile) = 2.92 $P = 0.25$
χ^2 (independence) = 108.80 $P < 0.0001$

[a] Six plants were identified as spontaneous cytoplasmic revertants and, therefore, were not included in the table

to date. These results suggest the existence of a relatively large introgressed segment flanking the *Fr* locus after six generations of backcrossing and emphasize the value of using bulked segregant analysis even when NILs are available for identifying *Fr*-linked DNA markers.

The mode of *Fr* action

In the mapping of the *Fr* gene we have grouped both fertile and semisterile F_2 segregants into one class to avoid any possibility of misclassification. However, there is generally a clear phenotypic distinction between fertile plants and semisterile plants, and we have observed 1 fertile: 2 semisterile: 1 sterile ratios for a number of F_2 segregating populations (data not shown). It was suspected that the semisterile phenotype may reflect the *Frfr* heterozygous genotype. The use of *Fr*-linked codominant DNA markers in the segregation analysis confirmed that *Fr* acts in a semidominant fashion. The pairwise cosegregations of the fertile phenotype with the *AA* genotype at the *Bng228* locus, semisterile with the *Aa* genotype and sterile with *aa* are shown in Table 4. Because *Bng228* is tightly linked to the *Fr* locus, it can be concluded from this table that a genotype of *FrFr* gives rise to fully fertile plants, *Frfr* to semisterile plants and *frfr* to fully sterile plants. These results indicate that one dose of the *Fr* allele is not sufficient to fully restore pollen fertility.

Discussion

Previous studies have shown that CMS-bean and its *Fr*-mediated restoration of pollen fertility represent a unique system in the studies of nuclear-mitochondrial interactions. Unlike most other CMS systems, *Fr* restores pollen fertility by eliminating the sterility-associated *pvs* mitochondrial sequence. This raises the interesting possibility that *Fr* may be involved in maintaining normal mitochondrial function by selectively eliminating mutations. Unfortunately, virtually nothing is known about how *Fr* effects this process. Molecular cloning of the gene will be essential to understand fully the molecular basis of *Fr* action. Based on the genetic information now available regarding *Fr* gene action and map location, a map-based cloning strategy should be feasible. In this study, we have constructed a relatively dense regional map encompassing the *Fr* locus, with some of the markers being tightly linked to *Fr*. *UBC487* (*R335E*) co-segregated with the *Fr* allele in a total of 125 F_2 plants, which corresponds to a maximum estimated recombination frequency of 0.0119 at 95% confidence probability. This marker should provide a useful starting point for the localization of the *Fr* locus.

In the original mapping population used to develop the bean map, it was suggested that linkage group K is the smallest of the 11 linkage groups, with 44 markers covering only 56.2 cM and 27 of them organized into four separate clusters (Vallejos et al. 1992). In this study, we detected a dramatic increase in the genetic distances among some markers. For example, *Bng14* and *Bng228*, originally demonstrating complete linkage, were 19 cM apart in the R351 BC_4F_2 mapping population (Fig. 4). This discrepancy was not unexpected. One of the parents (XR235-1-1) involved in the development of the original population used to construct the common bean linkage map contained *P. coccineus* introgressions (Vallejos et al. 1992). Recombination suppression due to this interspecific introgression may account for the observed low recombination frequencies and marker clusters in some regions of the linkage map. Alternatively, the *Fr* locus may represent a recombination "hot spot", a region of unusually high frequency recombination. Hot spots for recombination have been observed in the genomes of a number of organisms (Grimm et al. 1989; Gao et al. 1990; Ponticelli and Smith 1992; Snoek et al. 1993).

The relationship observed between codominant phenotypes (fertile/semisterile/sterile) and the codominant genotypes of *Fr*-linked markers (Table 4) provides important evidence that the *Fr* allele acts in a semidominant fashion. This result has interesting implications for how the *Fr* locus may be involved in altering the mitochondrial population. Physical mapping of the CMS-common bean mitochondrial genome has suggested that, unlike most other plant species, this particular mitochondrial genome contains three autonomous, inter-recombining chromosomes, one of which contains the sterility-inducing *pvs* sequence. The *pvs*-containing form is apparently absent from the mitochondrial genome of restored fertile or revertant plants (Janska and Mackenzie 1993). The dispensability of the *pvs*-containing molecule, together with evidence from other systems demonstrating the phenomenon of mitochondrial fusion within a population (Hoffman and Avers 1973; Belliard et al. 1979; Boeshore et al. 1983; Kawano et al. 1993) suggests that the mitochondrial population in CMS-bean could exist in a heteroplasmic state; that is, the mitochondrial population in a cell may be composed of a mixture of both normal (*pvs*−) and dysfunctional (*pvs*+) organelles. This hypothesis of mitochondrial heteroplasmy is supported by the observation of cytoplasmic reversion, a spontaneous phenomenon resulting in the loss of the *pvs*-

containing molecule (Janska and Mackenzie 1993). Surprisingly, the mitochondrial population in CMS-Sprite has been maintained relatively stably through many generations, with only low-frequency incidence of spontaneous reversion. Such a stable condition of heteroplasmy in CMS-Sprite mitochondria raises the question of why the loss of *pvs* does not occur more frequently as the result of random mitochondrial sorting to homoplasmy. One possible explanation is that the *fr* allele plays a role in maintaining the balance of the two mitochondrial types. On the basis of our observations to date, one possible role of the *Fr* allele is to stimulate mitochondrial segregation in favor of the normal (*pvs*−) mitochondrial type. A heterozygous genotype (*Frfr*) at the *Fr* locus produces only partial restoration of pollen fertility, suggesting that one dose of the *Fr* allele is not sufficient to completely eliminate *pvs* from the mitochondrial population. It will now be important to determine whether this is solely the result of gene dosage effect or the result of opposing influences effected by *Fr* and *fr* alleles when combined.

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